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Long noncoding RNA HOTTIP overexpression: A potential prognostic biomarker in prostate cancer

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ABSTRACT

HOXA transcript at the distal tip (HOTTIP) is a long noncoding RNA (lncRNA), which is > 200 nucleotides in length. HOTTIP expression has been demonstrated to play a crucial oncogenic role in cancer pathogenesis, and is said to be associated with poor human cancer prognosis. In prostate cancer, HOTTIP has been identified as an oncogene, but its clinicopathologic significance remains unclear. Array-based qRT-PCR was used to investigate lncRNA levels in 10 pairs of prostate cancer tissues and non-neoplastic parenchyma. Tissue microarray (TMA) was constructed using a total of 70 surgically resected prostatic adenocarcinoma tissues obtained from the Korea University Anam Hospital from 2009 to 2013. HOTTIP expression was determined by RNA *in situ* hybridization (ISH) and was correlated with clinicopathologic features. Increased HOTTIP expression was observed in all available prostate cancer tissue specimens compared with that in paired normal tissue. High HOTTIP expression was positively associated with bad clinicopathologic features, including higher pathologic T stage ($p < 0.001$), presence of extraprostatic extension ($p < 0.001$), seminal vesicle invasion ($p < 0.001$), perineural invasion ($p < 0.001$), and the tumor involvement of resection margin ($p = 0.044$). In particular, significantly increased HOTTIP expression was observed in specimens from patients in the high or very high-risk group, according to the 2018 National Comprehensive Cancer Network (NCCN) guidelines ($p < 0.001$). Also, patients with high HOTTIP expression showed poorer overall survival than those with low expression. In conclusion, we analytically validated the poor prognostic significance of HOTTIP overexpression and its association with bad clinicopathologic features, and present HOTTIP as a potential prognostic biomarker in prostate cancer.

1. Introduction

Due to recent technological advances with respect to researching human genomes, including next-generation sequencing, it has been evidenced that the human genome is much more complex than previously expected. Long noncoding RNA (lncRNA) is a group of non-protein-coding RNA molecules, > 200 nucleotides in length [1]. An increasing number of cancer-related lncRNAs are being recognized as significant contributors to human carcinogenesis and malignancy and as potential oncogenes or tumor suppressors [2]. However, few studies have functionally characterized and clinically validated the importance of dysregulated lncRNAs *in vitro* [3–6].

The lncRNA, 'HOXA transcript at the distal tip' (HOTTIP), has been identified to be associated with the human homeobox loci [4], and coordinates the activation of multiple 5' homeobox A genes *in vivo* [7]. HOTTIP expression has been demonstrated to regulate genes by various mechanisms and to be significantly upregulated in many human cancers; so it seems that it plays a crucial role in cancer pathogenesis [7–9]. Many articles have suggested that HOTTIP expression is associated with poor prognosis of human cancers, and implied its important oncogenic role in cancer pathogenesis [8,10,11]. Recently, more studies have revealed HOTTIP as a potential prognostic biomarker for various human

Abbreviations: AJCC, American Joint Committee on Cancer; CT, cycle threshold; FFPE, formalin-fixed, paraffin-embedded; HOTTIP, HOXA transcript at the distal tip; ISH, *in situ* hybridization; ISUP, International Society of Urological Pathology; lncRNA, long noncoding RNA; NCCN, National Comprehensive Cancer Network; PSA, prostate specific antigen; qRT-PCR, quantitative real-time polymerase chain reaction; TMA, tissue microarray; WHO, World Health Organization

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cancers [2,12–17].

Prostate cancer is one of the common causes of cancer morbidity and mortality among the male population worldwide [18]. The initiation and aggravation of prostate cancer include complex and dynamic biological processes, involving various genomic and epigenetic changes. Some studies have demonstrated that aberrant expression of individual lncRNAs in prostate cancer is related to disease progression, and have attempted to investigate lncRNAs specific to prostate cancer [3,19].

The functional role of HOTTIP, as an oncogene, has been identified [4], but its clinicopathologic significance remains unclear. This study was aimed to investigate HOTTIP expression in human prostate cancer tissues, and clarify the association between HOTTIP expression and clinicopathologic features.

2. Material and methods

2.1. Sample collection and tissue microarray construction

Tissue microarray (TMA) was constructed with prostate cancer tissue specimens from 70 patients. These samples had been collected by radical prostatectomy at the Korea University Anam hospital from 2009 to 2013. Patients who were diagnosed with other cancers or who received other treatment before operation were excluded. Total 70 cases were selected, which had enough formalin-fixed paraffin embedded tissue block to make tissue microarray. Patients classified as pathologically acinar adenocarcinoma were selected according to the World Health Organization (WHO) / International Society of Urological Pathology (ISUP) 2014 classification, and two pathologists reviewed the slides to document pathological features. Staging was classified according to the American Joint Committee on Cancer (AJCC) 8th edition and pathological findings, such as extraprostatic extension, seminal vesicle invasion, lymphatic invasion, vascular invasion, perineural invasion, Gleason's score, grade group according to the ISUP 2014 grading system [20] were investigated. The National Comprehensive Cancer Network (NCCN) risk group was divided into two groups according to the 2018 NCCN guidelines [21], and the high or very high risk group was classified as high risk group, very low, low and intermediate group as low risk group. In addition, age, serum prostate specific antigen (PSA) level, additional post-operation therapy, recurrence and death, and the last outpatient date were investigated. Two tissue cores (each 3 mm in diameter) were obtained from formalin-fixed, paraffin-embedded (FFPE) tissue blocks of prostate cancer specimens to construct tissue microarray. This study was approved by the Institutional Review Boards of Korea University Anam Hospital (IRB No. 2018AN0108).

2.2. RNA purification from fresh tissue

Array based quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was used to investigate lncRNAs levels in 10 pairs of high-risk prostate cancer tissues and non-neoplastic parenchyma. Five sections of fresh tissue, each, 10 μm thick and with ~100 mm^2 surface area, were used for total RNA extraction. Tissue sections were completely lysed with 0.8 mL Trizol reagent (Invitrogen, Carlsbad, CA), incubated at room temperature for 10 min. With an additional 0.2 mL chloroform, samples were vortexed and centrifuged for 15 min at 14,000 rpm. The aqueous phase was transferred to a new 1.5 mL tube, and ethanol precipitation followed. After suspension in 20 μL of RNase-free deionized water, the quality and quantity of isolated total RNA were measured using an Eppendorf BioPhotometer plus spectrophotometer (Eppendorf, Hamburg, Germany). Purified total RNA samples were stored at −80 °C before use.

2.3. Quantitative real-time polymerase chain reaction

Expression profiling of HOTTIP was performed using the LncProfiler™ qPCR Array Kit (System Biosciences, Mountain View, CA) according to the manufacturer's instructions. Real-time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix 2× (Thermo Scientific, Pittsburgh, PA) and the cycle number at which the reaction crossed a cycle threshold (CT) was determined for each gene. Raw CT values were normalized using a median CT value (ΔCT = CTlncRNA − CTmedian). The relative amount of HOTTIP in prostate cancer tissue relative to normal tissue (fold change) was described using the equation 2-ΔΔCT where ΔΔCT = ΔCTprostate cancer − ΔCTnormal tissue. Tail and tag cDNA were synthesized according to the manufacturer's protocols and real-time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix 2×. HOTTIP expression levels were evaluated using a comparative ΔCT method.

2.4. Single color RNA in situ hybridization

We performed single color RNA *in situ* hybridization (ISH) in accordance with the manufacturer's instructions through the RNAscope® 2.0 HD Red Chromogenic Reagent Kit (Advanced Cell Diagnostics, Newark, CA, USA). TMA sections of 4 μm-thick FFPE samples were used for ISH. The slides were baked in a dry oven for 1 h at 60 °C. After deparaffinization, dehydration, and air-drying slides, hydrogen peroxide was applied for 10 min at room temperature, and the slides were submerged in boiling pre-treatment solution for 15 min. Afterwards, Protease Plus was applied and the slides were incubated at 40 °C for 30 min. The target probes, including positive and negative controls, were hybridized for 2 h at 40 °C using a HybEZ™ Oven (Advanced Cell Diagnostics), and Amplifier 1 (30 min), Amplifier 2 (15 min), Amplifier 3 (30 min), and Amplifier 4 (15 min) at 40 °C. Amplifier 5 (30 min) and Amplifier 6 (15 min) were amplified at room temperature. After that, Fast Red substrate were added to enable visualization of RNA molecules as red chromogenic dots.

The probes used were Hs-HOXA transcripts at the distal tip, Hs-peptidyl-prolyl *cis*-trans isomerase B (housekeeping gene cyclophilin B, positive control), and DapB (probe-targeting bacterial gene dapB, negative control). The probe against Hs-HOTTIP RNA (Catalog number 400133, Advanced Cell Diagnostics) were designed using custom software. GenBank accession number was NR_037843.2, number of probe pairs was 20, and targeting probe regions was 388-1386. For probe specificity, first, all individual Z probes in the target probe set have been screened against all other known RNA molecules in the genome/transcriptome by the BLAST algorithm to ensure they do not have significant homologies to non-target sequences. Secondly, the specificity of signal detection is further ensured by the double Z probe strategy which requires a probe pair (ZZ) to simultaneously bind to the target in order to generate signal. Unspecific binding of single Z probes will not yield detectable signals. Since all RNAscope probes are designed by applying the same screening criteria and the assays run at the same stringent conditions (hybridization and washing conditions), a similar level of high specificity is expected for all probes. In addition, during the assay, a negative control probe (bacterial gene dapB) is run in parallel with the same sample to assess any nonspecific background in a particular run/sample.

Analyze the number of red dots in 1 HPF with the highest expression in each core, which means two high-power fields per case. Quantifying the number of points through the software, image J, and determining the cut-off value through a ROC curve, HOTTIP expression was divided into low and high expressions based on 237 dots.

2.5. Statistical analysis

Statistical analysis was conducted through the SPSS software (version 20.0, IBM, Boston, MA, USA). Chi-squared test was used to

Table 1
Clinicopathologic characteristics of tissue microarray samples with HOTTIP expression.

	N	HOTTIP score		p-value
		Low	High	
2018 NCCN risk group				< 0.001
Low-risk group	36 (51.4%)	31 (44.3%)	5 (7.1%)	
High or very high-risk group	34 (48.6%)	15 (21.4%)	19 (27.1%)	
Pathologic staging				< 0.001
T2	48 (68.6%)	39 (55.7%)	9 (12.9%)	
T3	22 (31.4%)	7 (10%)	15 (21.4%)	
2014 ISUP Grade group				0.301
Group 1 (Score < 6)	18 (25.7%)	15 (21.4%)	3 (4.3%)	
Group 2 (Score 3 + 4 = 7)	37 (52.9%)	23 (32.9%)	14 (20%)	
Group 3 (Score 4 + 3 = 7)	9 (12.9%)	4 (5.7%)	5 (7.1%)	
Group 4 (Score 8)	4 (5.7%)	3 (4.3%)	1 (1.4%)	
Group 5 (Score 9-10)	2 (2.9%)	1 (1.4%)	1 (1.4%)	
Extraprostatic extension				< 0.001
Absent	48 (68.6%)	39 (55.7%)	9 (12.9%)	
Present	22 (31.4%)	7 (10%)	15 (21.4%)	
Seminal vesicle invasion				< 0.001
Absent	58 (82.9%)	44 (62.9%)	14 (20%)	
Present	12 (17.1%)	2 (2.9%)	10 (14.3%)	
Lymphatic invasion				0.425
Not identified	66 (94.3%)	42 (60%)	22 (31.4%)	
Present	4 (5.7%)	2 (2.9%)	2 (2.9%)	
Venous invasion				N/A
Not identified	70 (100.0%)	46 (65.7%)	24 (34.3%)	
Present	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Perineural invasion				0.007
Not identified	33 (47.1%)	27 (38.6%)	6 (8.6%)	
Present	37 (52.9%)	19 (27.1%)	18 (25.7%)	
Resection margin				0.044
Free	29 (41.4%)	23 (32.9%)	6 (8.6%)	
Involvement	41 (58.6%)	23 (32.9%)	18 (25.7%)	
Score 5				0.342
Not identified	53 (75.7%)	36 (51.4%)	17 (24.3%)	
Present	17 (24.3%)	10 (14.3%)	7 (10%)	
Serum PSA level				0.232
Increased (> 4 ng/mL)	63 (90%)	40 (57.1%)	23 (32.9%)	
Normal (≤ 4 ng/mL)	7 (10%)	6 (8.6%)	1 (1.4%)	
Age (years)				0.249
60 <	46 (65.7%)	32 (45.7%)	14 (20%)	
60 ≥	24 (34.3%)	14 (20%)	10 (14.3%)	
Recur				0.032
Recurred	11 (15.7%)	4 (5.7%)	7 (10%)	
Not recurrence	59 (84.3%)	42 (60%)	17 (24.3%)	
Additional therapy after operation				< 0.001
No treatment	38 (54.3%)	32 (45.7%)	6 (8.5%)	
Hormonal therapy	32 (45.7%)	14 (20.0%)	18 (25.8%)	

correlate clinicopathologic factors with lncRNA expression. The survival curves were plotted using the Kaplan-Meier method and the log-rank test. Cox proportional hazard regression models were used to analyze the significance of lncRNA ISH expression and clinicopathologic variables. P-values < 0.05 were considered statistically significant.

3. Results

3.1. Clinicopathologic characteristics of the cases

Clinicopathologic data were collected retrospectively, and the findings are shown in Table 1. The median age was 64 years (range: 49–76). The average serum PSA level was 11.35 ng/mL (range: 3.34–45.91 ng/mL). 34 cases (48.6%) were classified as high-risk or very high-risk according to the 2018 NCCN guidelines, while 36 cases (51.4%) were low-risk. A total of eighteen cases (25.7%) had a Gleason score 6 or less, 46 cases (65.7%) had a Gleason score 7, and 6 cases (8.6%) had a Gleason score 8 or more. Pathologic staging was T2a in 3 cases (4.3%), T2b in 1 case (1.4%), T2c in 44 cases (62.9%), T3a in 10 cases (14.3%) and T3b in 12 cases (17.1%). Lymphatic invasion was identified in 4 cases (5.7%), perineural invasion in 37 cases (52.9%), and none had venous invasion. Twenty-nine cases (41.4%) had tumor-free resection margins, while 41 cases (58.6%) had resection margin involvement. According to the ISUP 2014 grading system, they were classified as follows: group 1 (Score < 6), 18 cases (25.7%); group 2, (Score 3 + 4 = 7), 37 cases (52.9%); group 3 (Score 4 + 3 = 7), 9 cases (12.9%); group 4 (Score 8), 4 (5.7%) case; and group 5 (Score 9–10), 2 cases (2.9%). There were 11 (15.7%) recurring cases in follow-up data, and none of them were cancer-related deaths. Disease-free survival was defined as the interval from the date of diagnosis to recurrence or last follow-up date, and averaged 87.2 months (26.8–122.4 months). 32 out of 70 patients (45.7%) received additional hormonal treatment after surgery.

3.2. HOTTIP expression by qRT-PCR

We performed qRT-PCR to investigate the expression level of lncRNA HOTTIP in prostate cancer and paired benign tissue of a high-risk group. A comparative ΔCT value of HOTTIP expression measured 2.54–9.77 (average 5.09). Although three samples were unavailable for lncRNA detection, all other seven specimens showed HOTTIP over-expression in prostate cancer tissue compared to normal tissue, implying oncogenic effect of HOTTIP expression.

3.3. HOTTIP expression by RNA in situ hybridization

The number of dots in HOTTIP was 61–876 per case (mean 222.7). High HOTTIP expression was found in 24 cases (34.3%), and low expression in 46 cases (65.7%) (Fig. 1).

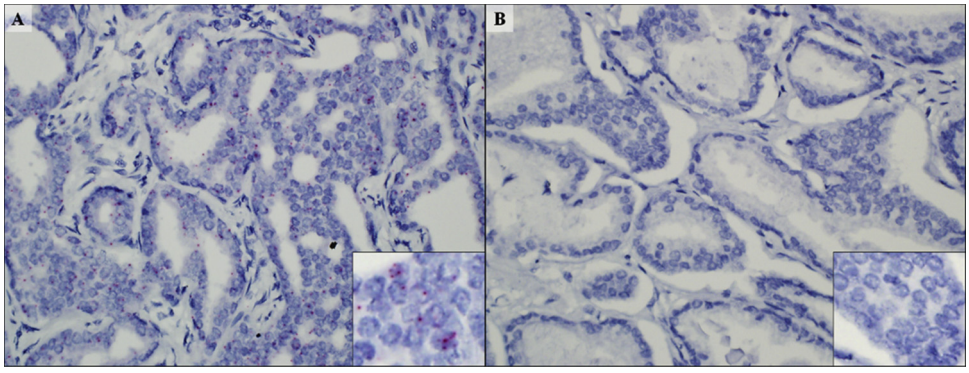

Fig. 1. The representative images of HOTTIP RNA *in situ* hybridization with (a) high expression and (b) low expression.

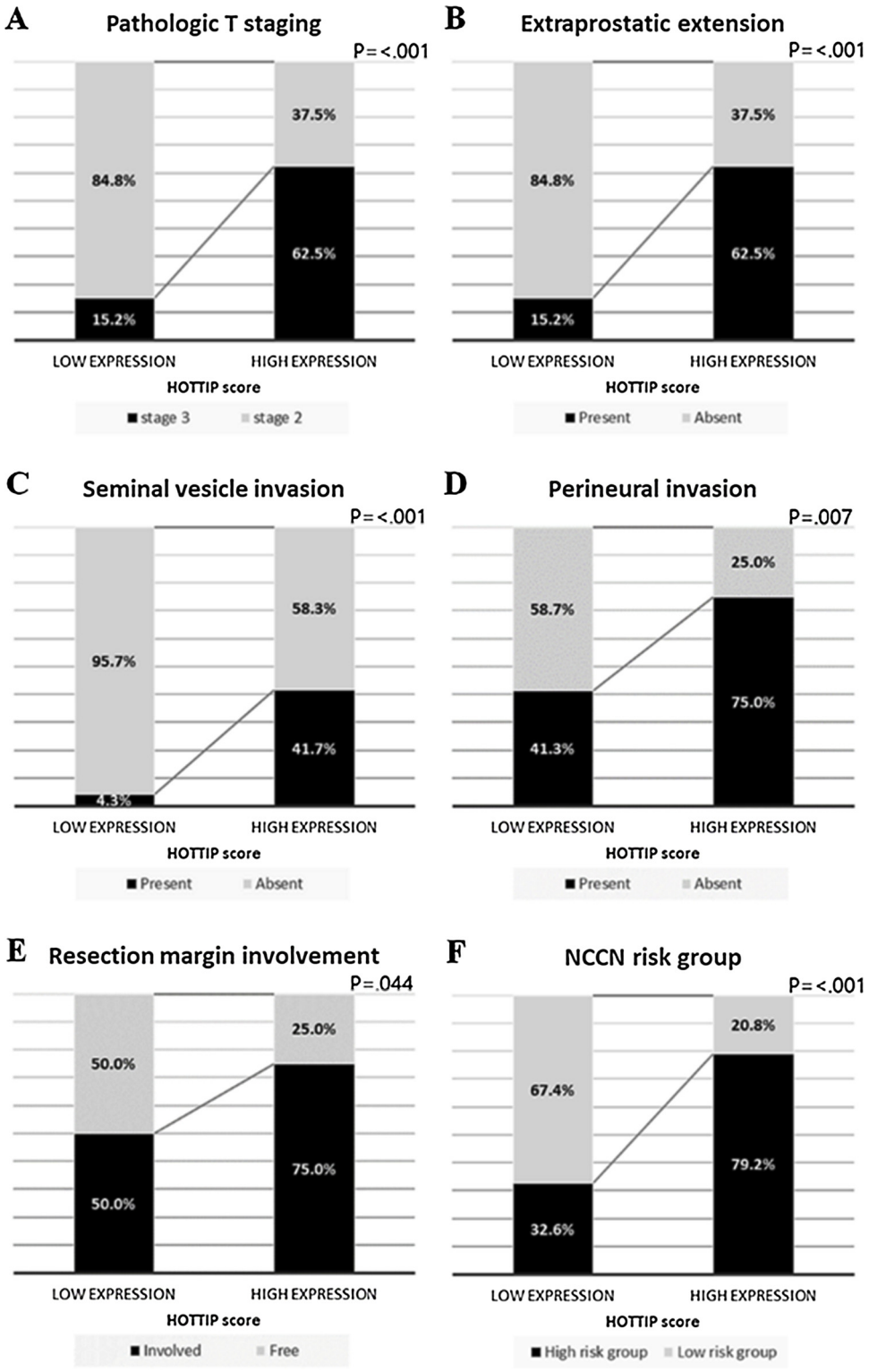

Fig. 2. Relationship between HOTTIP expression and clinicopathologic factors, including (a) pathologic T staging, (b) extraprostatic extension, (c) seminal vesicle invasion (d) perineural invasion, (e) tumor involvement of resection margin, and (f) NCCN risk group.

3.4. Relationship between HOTTIP expression and clinicopathologic factors

We investigated the association between HOTTIP expression and clinicopathologic features (Fig. 2, Table 1). High HOTTIP expression was positively associated with bad clinicopathologic features, including higher pathologic T stage ($p < 0.001$), presence of extraprostatic extension ($p < 0.001$), seminal vesicle invasion ($p < 0.001$), perineural invasion ($p < 0.001$), and tumor involvement of resection margin ($p = 0.044$). Increased HOTTIP expression was significantly identified in specimens from patients in the high- or very high-risk groups, according to 2018 NCCN guidelines ($p < 0.001$). No significant differences were observed for the other factors.

The specimens with high HOTTIP expression are associated with high risk clinicopathologic features, so more patients with specimens of

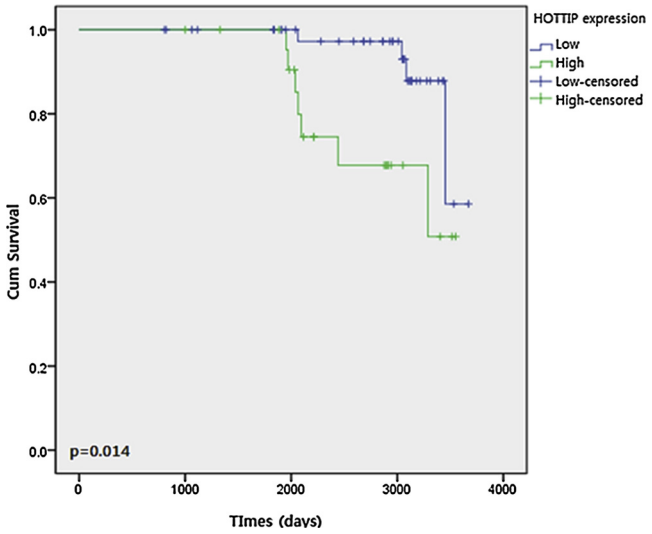

Fig. 3. Prognostic significance of HOTTIP expression.

high expression received additional hormonal therapy than patients with specimens of low expression. There was a statistically significant association between post-operative treatment and HOTTIP expression level. However, the treatment or otherwise does not affect the recurrence rate. (data not shown).

3.5. The prognostic value of HOTTIP overexpression

To evaluate the prognostic value of HOTTIP expression, we performed survival analysis, using the Kaplan-Meier analysis and log-rank test. Patients with high HOTTIP expression showed poor overall survival than those with low expression (log-rank = 5.988, p = 0.014, Fig. 3). The mean overall survival time for high and low HOTTIP expression groups were 101 and 116 months, respectively. The Cox proportional hazards regression analysis model showed that unfavorable prognostic factors for recurrence include higher grade group (p = 0.048, HR = 3.887, 95% CI = 1.009–14.970), lymphatic invasion (p = 0.047, HR = 3.903, 95% CI = 1.017–14.980), serum PSA level (p = 0.013, HR = 1.076, 95% CI = 1.015–1.141) and high HOTTIP overexpression (p = 0.014, HR = 4.221, 95% CI = 1.213–14.694).

4. Discussion

Protein-encoding genes account for only 1.5% of the total genome, while the rest, called noncoding regions, do not encode proteins. As many biological processes at the transcriptional and posttranscriptional level cannot be explained only by protein-coding genes, it has been suggested that lncRNAs participate in various cellular processes and their dysregulation contributes to the development of various diseases, especially tumor development [8,22].

HOTTIP is a lncRNA found to have significant oncogenic effect in various human cancers, as it coordinates the activation of multiple 5' HOXA genes *in vivo* [14,23]. HOTTIP induces chromatin modification to regulate gene expression, similar to other lncRNAs. When transcribed, HOTTIP directly bind the adapter protein WDR5 inducing an open DNA-chromatin configuration to target WDR5/MLL complexes, thus driving histone H3 lysine 4 trimethylation and regulating the transcription of HOXA13 [14]. HOXA13 has been known to have a crucial role in tumorigenesis of the liver, bladder and esophageal cancer [4]. In prostate cancer, Zhang et al. [4] demonstrated that knocking down of HOTTIP or HOXA13 resulted in reduced cell proliferation, implying their role in development and aggravation of prostate cancer. They demonstrated that knockdown of HOTTIP in prostate cancer cell lines reduced cell viability, induced cell apoptosis, and G0/G1 phase arrest, and inhibited the G1/S cell cycle transition. Also, Jiang et al. [18] demonstrated that its knockdown enhances prostate cancer cell chemosensitivity to cisplatin. To our knowledge, HOTTIP has been identified as an oncogene in prostate cancer cells [4,18], and Yang et al. [24] identified HOTTIP was associated with TNM stage and tumor size, but its prognostic significance in prostate cancer is poorly understood.

Our study found that HOTTIP is highly overexpressed in high-risk prostate cancer compared to that in benign samples. Also, we identified that high HOTTIP expression is associated with bad clinicopathologic features, including pathologic T stage, presence of extraprostatic extension, seminal vesicle invasion, perineural invasion, and tumor involvement of resection margin. Prostate cancer patients with high HOTTIP expression also had poor prognosis compared to those with low expression, and high HOTTIP expression was an independent unfavorable prognostic marker for recurrence. This means that HOTTIP overexpression is associated with poor prognosis and bad clinicopathologic features, supporting its potential as a prognostic biomarker.

Importantly, we observed significantly increased HOTTIP expression in specimens from patients in the high- or very high-risk groups, according to the 2018 NCCN guidelines [22]. Considerations for localized prostate cancer treatment options based on the 2018 NCCN guideline include Gleason score, initial serum PSA level, and tumor T stage evaluations. Active surveillance can be an option for patients in the low-risk group, but radical prostatectomy or radiotherapy will be more advantageous in high-risk group patients. To develop a more effective treatment plan, it would be important to find a novel diagnostic and prognostic biomarker based on molecular methods [25]. Although several lncRNAs have been reported as promising prognostic biomarkers [26–28], most of them have not been validated enough to be used in clinical settings. This study was designed to analytically validate the clinicopathologic significance of HOTTIP, suggesting potential as a prognostic biomarker.

Ideal biomarkers should allow noninvasive targeting and be found in easily accessible samples, including body fluids, such as serum and urine. So, using circulating RNA or DNA species floating in the body fluids will be a new direction for biomarkers. In the case of prostate cancer, considering that urine passes through the prostate anatomically, clinical possibilities of lncRNA detection in urine samples can be considered. However, further detailed research will be needed to assess this possibility.

The current method for clinical detection of prostate cancer is serum PSA testing, but false positive results could lead to unnecessary biopsies [29] and the aggressive status is not correlated to the serum PSA level. Therefore, many effective diagnostic biomarkers have been identified *via* various approaches, and lncRNA could be promising candidates. For example, prostate cancer antigen 3 (PCA3), the first lncRNA found in prostate cancer [30], is the most specific prostate cancer biomarker studied and urine PCA3 level is a potential diagnostic biomarker [29,31–33]. Hence, the Progensa urinary PCA3 assay (Hologic, Marlborough, Massachusetts) was approved by the US Food and Drug Administration (FDA) in 2012. However, PCA3 was found to have only diagnostic utility, and there is still controversy over its prognosis as a predictor [29]. On the other hand, as this study showed that increased HOTTIP expression is associated with high risk clinicopathologic features and recurrence, a new insight is proposed that HOTTIP is a lncRNA with potential as a biomarker for prognosis of prostate cancer, which is strength of this study. There are some limitations to this study. First, the patient group for TMA did not include patients who died of prostate cancer. Second, the RNA ISH results were not validated by other molecular methods. Only few studies used the ISH method to determine the amount of lncRNA in FFPE clinical samples [23,34,35]. Since FFPE is one of the most widely used and effective tissue storage methods in the world, clinical studies through FFPE enable long clinical follow-up of patients. We could demonstrate the oncogenic role of HOTTIP in prostate cancer using RNA ISH as in other reports, so it implies the possibility of using ISH methods for lncRNA analysis.

5. Conclusions

In conclusion, we analytically validated the poor prognostic significance and association with bad clinicopathologic features of HOTTIP overexpression, and present it as a potential prognostic biomarker in prostate cancer.

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Declaration of Competing Interest

None.

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